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Spontaneous proliferation of memory (CD45RO$^+$) and naive (CD45RO$^-$) subsets of CD4 cells and CD8 cells in human T lymphotropic virus (HTLV) infection: distinctive patterns for HTLV-I *versus* HTLV-II

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SUMMARY

Spontaneous lymphocyte proliferation (SLP) *in vitro* is a characteristic feature of about 50% of individuals infected with HTLV-I or HTLV-II. Both CD4 cells and CD8 cells contribute to SLP in HTLV-I infection, whereas SLP in HTLV-II infection is usually restricted to CD8 cells. In this study, we asked if SLP was restricted to the memory (CD45RO$^+$) cell subset of CD4 and CD8 cells in HTLV infection. Purified CD4 and CD8 cells were separated into CD45RO$^+$ and CD45RO$^-$ populations by a modified panning technique, and spontaneous proliferation (SP) of the cell subsets was assessed. For all five HTLV-I-infected persons whose mononuclear cell cultures were SLP$^+$, only CD45RO$^+$ cells, but not CD45RO$^-$ cells, within CD4 and CD8 subsets showed SP. In contrast, five of six SLP$^+$ HTLV-II$^+$ individuals showed SP in both the CD45RO$^+$ and the CD45RO$^-$ subsets of CD4 cells, and 10 of 12 SLP$^+$ HTLV-II$^+$ individuals showed SP of both the CD45RO$^+$ and CD45RO$^-$ subsets of CD8 cells. Polymerase chain reaction studies showed that proviral genome was generally present in both CD45RO$^+$ and CD45RO$^-$ subsets of CD4 and CD8 cells, regardless of HTLV type and SP activity. These findings show that SP of both CD4 and CD8 cells in HTLV-I infection is usually restricted to CD45RO$^+$ memory cells, whereas in HTLV-II infection, both CD45RO$^+$ memory and CD45RO$^-$ naive subsets of CD4 and CD8 cells may exhibit SP. It thus appears that HTLV-I infection and HTLV-II infection exhibit distinctive dysregulatory effects on memory and naive T cell subpopulations.

Keywords HTLV-I HTLV-II spontaneous proliferation memory T cells naive T cells

INTRODUCTION

HTLV types I and II are closely related human retroviruses, yet they exhibit distinctive disease associations. HTLV-I infection is associated with adult T cell leukaemia, a neurologic disorder designated HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and some cases of inflammatory arthritis and uveitis [1–4]. HTLV-II infection, in contrast, has only been associated with a few cases of a neurologic disorder similar to HAM/TSP [5,6]. The factors regulating disease development in these infections remain unclear; only a very low proportion of infected individuals ever manifest any of these clinical abnormalities [7,8].

Peripheral blood mononuclear cells (PBMC) from $\approx 50\%$ of asymptomatic individuals infected with HTLV-I or HTLV-II exhibit spontaneous proliferation (SP) of lymphocytes during *in vitro* culture [9–11]. However, almost all individuals with HAM/TSP [12,13] show lymphocyte SP. The SP response is monocyte-dependent, and requires both IL-2 production and IL-2 receptor expression [14–17]. The activation mechanism apparently involves transactivation of the IL-2 and IL-2 receptor genes by the virus-associated Tax gene product [18].

Limited information is available on the lymphocyte subsets undergoing activation as part of the SP response. Flow cytometric analyses revealed that the SP response is characteristic of T cells, but not B cells in both HTLV-I and HTLV-II infection [15]. In HTLV-I infection, both purified CD4 cells and purified CD8 cells undergo SP, with the CD4 subset typically exhibiting a more robust response. In contrast, for HTLV-II infection, 75% of SP$^+$ individuals show restriction of SP to the purified CD8 cell subset; the remaining 25% demonstrate SP in both CD4 cells and CD8 cells [14]. The capacity for SP does not simply reflect subset infection with HTLV; CD4 cells from HTLV-II-infected individuals showing only CD8 cell SP nevertheless carry provirus, as do both CD4 cells and CD8 cells from SP$^-$ individuals infected with either HTLV type [14]. This lack of association between SP and provirus in a specific T cell

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subset led us to examine the possibility that SP was restricted to memory or naive T cell subsets.

Memory T cells, generated by antigenic activation, express high levels of CD45RO, a low molecular weight isoform of the CD45 molecule [19,20]. Acquisition of high levels of CD45RO is associated with functional maturation of T cells; CD45RO$^+$ CD4 cells show an increased proliferative response to soluble antigen, and the CD45RO$^+$ CD8 cell subset contains most of the cytotoxic T cell activity associated with the CD8 cell subset [20]. A population of T cells with a lower cell surface density of CD45RO (CD45RO^{dim}) is also found in the circulation, and appears to represent recently activated cells in the process of functional maturation [21,22].

In the study presented here, we sought to dissect further which T cell subsets exhibit SP *in vitro* in association with HTLV infection. Comparison of the SP capacity of CD45RO$^+$ and CD45RO$^-$ subsets of CD4 cells and CD8 cells from HTLV-infected individuals provides further evidence for differences in the cell subsets with SP capacity in HTLV-I *versus* HTLV-II infection.

PATIENTS AND METHODS

Study subjects

All study subjects were Los Angeles participants in the Retroviral Epidemiology in Donors Study (REDS) sponsored by the National Heart, Lung, and Blood Institute. The study group included six SLP$^+$ HTLV-I-infected individuals, 18 SLP$^+$ HTLV-II-infected individuals, and six uninfected controls. Blood specimens were taken as part of a routine biannual visit, after informed consent was obtained. Because the goal of the study was to assess the SP response of T cell subsets, we biased our study cohort toward spontaneous lymphocyte proliferation (SLP)$^+$ HTLV-infected individuals. The greater number of HTLV-II-infected subjects *versus* HTLV-I-infected subjects studied reflects the predominance of HTLV-II infection in the Los Angeles blood donor population [14].

Cell purification

PBMC, monocytes, purified CD4 cells, and purified CD8 cells were obtained as previously described [14,15]. Briefly, PBMC obtained by density gradient centrifugation of heparinized venous blood were incubated in plastic culture dishes to allow monocyte adherence. Non-adherent cells were treated with anti-CD14 and goat anti-mouse immunoglobulin-coated magnetic beads to remove residual monocytes. CD4 cells and CD8 cells were then captured using magnetic beads coated with subset-specific MoAbs (Dynal, Oslo, Norway); captured cells were released from beads using detach-a-bead reagent (Dynal). Plastic-adherent monocytes were recovered using a disposable cell scraper.

After reserving enough cells to assess SP capacity of unfractionated cells, CD4 cells and CD8 cells were treated with a saturating concentration of anti-CD45RO MoAb (Becton Dickinson Immunocytometry Systems, San Jose, CA) (10 μl/1 $\times$ 10^6 cells) at room temperature for 30 min. After washing with PBS containing 5% pooled human serum (PBS–PHS), the cells were resuspended in 4 ml PBS–PHS and added to a culture flask covalently coated with goat anti-mouse immunoglobulin (Applied Immune Sciences, Santa Clara, CA). The flask was centrifuged for 3 min at 150 ***g***; the flask orientation was then reversed and the centrifugation step repeated. Non-bound cells were harvested, centrifuged, resuspended at a concentration of 5 $\times$ 10^5/ml in culture medium (RPMI 1640 containing antibiotics and 10% PHS), and used as a CD45RO$^-$ cell population. Following the addition of 4 ml PBS–PHS to the flask, bound cells were harvested using a disposable cell scraper. The cells were washed and resuspended in culture medium as above, and used as a CD45RO$^+$ cell population.

Cell culture

SLP by PBMC preparations was assessed in 7-day cultures as previously described [14,15]; results > 3100 ct/min were considered SLP$^+$. These data were then used to assign individual HTLV-infected subjects to the SLP$^+$ or SLP$^-$ group. Purified CD4 cells, CD8 cells, CD45RO$^+$ CD4 cells, CD45RO$^-$ CD4 cells, CD45RO$^+$ CD8 cells, or CD45RO$^-$ CD8 cells were cultured with autologous monocytes (50 000 lymphocytes plus 10 000 monocytes) in triplicate microtitre wells for 7 days at 37°C in 5% CO_2. DNA synthesis was then assessed by ^{3}H-thymidine incorporation as described [14,15]. In some experiments, CD4 cells or CD8 cells treated with anti-CD45RO were cultured with autologous monocytes in an identical fashion.

Flow cytometry

The purity of CD4 cells and CD8 cells were assessed by a routine flow cytometric method using PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 (Becton Dickinson Immunocytocytometry Systems) and a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems) as described [14,15]. Flow cytometry was also used to assess the purity of CD45RO$^+$ and CD45RO$^-$ subsets of CD4 cells and CD8 cells. Two approaches were used. In the first approach, the cell subsets were treated with FITC-conjugated goat anti-mouse immunoglobulin; untreated CD4 cells or CD8 cells served as negative control. The second approach was based on the relationship of CD45RA expression to CD45RO expression; thus, both subsets were treated with FITC-conjugated anti-CD45RA MoAb (Becton Dickinson Immunocytometry Systems).

Polymerase chain reaction studies to detect proviral genome

When a minimum of 5 $\times$ 10^5 cells of a given purified cell subset remained after establishing cell cultures, the cells were pelleted, frozen, and subsequently analysed by polymerase chain reaction (PCR) studies to detect proviral genome [23]. Briefly, purified lymphocyte subset populations were lysed in 0·125 ml of PCR lysis buffer (10 mM Tris–HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·45% NP-40, 0·45% Tween-20, and 0·01% w/v gelatin) containing 20 μg Proteinase K (2 μl of 10 mg/ml stock). The cell suspension was incubated at 56°C for 1 h followed by incubation at 95°C for 15 min to inactivate the proteinase K, and 25 μl of cell lysate (1·0 μg sample DNA) were added to 75 μl of the reagent mixture to give a final amplification mixture of 200 mM of each dNTP, 100 ng each of primers, and 2·5 U of recombinant *Taq* polymerase in PCR buffer. The amplification conditions followed were: denaturatuion at 94°C for 5 min; annealing for 1 min at 55°C and extension at 72°C for 1 min, for 45 cycles, followed by a final 7-min extension at 72°C. The *pol* region was amplified, using the primers SK110/111 for the first round of PCR, followed by specific hybridization with probe SK188, resulting in a 185-base-pair (bp) product. Recently, an enzyme oligonucleotide-based amplification and hybridization procedure has been developed using SK110/111 primers, which results in greater sensitivity of HTLV-II detection. This assay was performed following the manufacturer's instructions (HTLV-II EOA; Cellular Products, Inc., Buffalo, NY).

RESULTS

Flow cytometric assessment of cell subsets

All CD4 cell and CD8 cell preparations were > 98% positive for the appropriate marker. CD45RO$^-$ cell preparations were always < 10% CD45RO$^+$ using FITC-conjugated goat anti-mouse immunoglobulin, whereas CD45RO$^+$ cell preparations were always > 90% CD45RO$^+$ (data not shown). As another measure of CD45RO subset purity, we capitalized on the relationship of CD45RA expression to CD45RO expression. We and others have shown that three populations of lymphocytes can be defined on the basis of CD45RO and CD45RA expression—CD45RO$^-$CD45RA^{bright}, CD45RO^{dim} CD45RA^{dim}, and CD45RO^{bright}CD45RA$^-$ [21,22]. Based on this relationship, we thus hypothesized that our CD45RO$^-$ subset should contain only CD45RA^{bright} cells, whereas our CD45RO$^+$ cell subset should include both CD45RA^{dim} and CD45RA$^-$ subsets. As shown in Fig. 1, this hypothesis was confirmed. The CD45RO$^-$ subset of both CD4 cells and CD8 cells consisted primarily of CD45RA^{bright} cells, whereas the CD45RO$^+$ subset of both CD4 cells and CD8 cells contained cells that were CD45RA^{dim} or CD45RA$^-$.

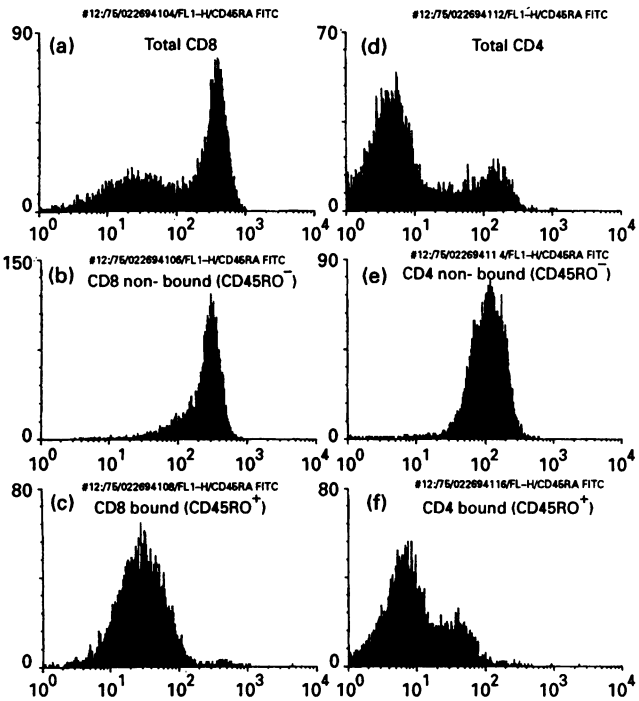

Fig. 1. CD45RA expression by subsets of CD8 cells and CD4 cells selected on the basis of CD45RO expression. Total (a,d) refers to anti-CD45RO-treated cells before incubation on a goat anti-mouse immunoglobulin-coated flask, non-bound (b,e) refers to treated cells not bound to the flask (CD45RO$^-$), and bound (c,f) refers to treated cells bound to the flask and recovered using a cell scraper (CD45RO$^+$). All cell subsets were then stained with FITC-anti-CD45RA and analysed by flow cytometry.

SP of naive and memory T cell subsets

Since antibodies to T cell surface structures may modulate SP [24], we wanted to ensure that anti-CD45RO did not interfere with the SP capacity of CD4 cells and CD8 cells. Table 1 shows that the SP response of anti-CD45RO-treated cells was not significantly different from that of untreated cells.

SP responses of CD45RO$^+$ and CD45RO$^-$ subsets of both CD4 cells and CD8 cells were examined in HTLV$^-$ controls (n = 6), HTLV-I/II$^+$ SLP$^-$ (n = 7), HTLV-I$^+$ SLP$^+$ (n = 5), and HTLV-II$^+$ SLP$^+$ (n = 12) individuals. ^{3}H-thymidine incorporation values were considered SP$^+$ if above the following critical values, which represent the mean of the six controls + 2 s.d.: CD45RO$^+$ CD4 = 967 ct/min; CD45RO$^-$ CD4 = 1274 ct/min; CD45RO$^+$ CD8 = 1224 ct/min; CD45RO$^-$ CD8 = 1285 ct/min. Analysis of the CD4 cell subsets demonstrated that all five HTLV-I$^+$ SLP$^+$ individuals showed SP of the CD45RO$^+$ cell subset only, and not the CD45RO$^-$ cell subset (Fig. 2). In contrast, five of the 12 HTLV-II$^+$ SLP$^+$ individuals (nos 19, 20, 26, 27 and 30) showed SP within both the CD45RO$^+$ and CD45RO$^-$ CD4 cell subsets; one HTLV-II$^+$ SLP$^+$ subject (no. 25) showed SP in the CD45RO$^-$ CD4 subset only, and the remaining six subjects showed no SP in either subset of CD4 cells. These six individuals (nos 21–24, 28 and 29) showed unfractionated CD4 cell SP < 1000 ct/min, as was observed in the control and HTLV-

Table 1. Influence of anti-CD45RO treatment on the spontaneous proliferation (SP) capacity of CD4 cells and CD8 cells*

	CD4 cells		CD8 cells	
Group (n)	Untreated	Anti-RO-treated	Untreated	Anti-RO-treated
Control (5)	753 ± 271	828 ± 626	977 ± 648	674 ± 318
I/II$^+$SLP$^-$ (7)	405 ± 194	546 ± 206	249 ± 212	339 ± 162
I$^+$SLP$^+$ (5)	52 284 ± 39 351	50 140 ± 34 201	22 307 ± 34 201	22 719 ± 38 723
II$^+$SLP$^+$ (10)	7612 ± 10 008	6431 ± 9799	53 479 ± 52 586	46 627 ± 48 015

*Results represent mean ct/min±1 s.d. No significant differences between treated and untreated cell types were observed (P > 0·05, paired t-test).

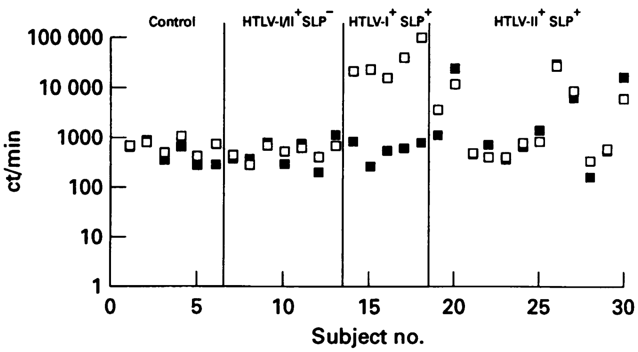

Fig. 2. Spontaneous proliferation (SP) activity of CD45RO⁻ and CD45RO⁺ subsets of CD4 cells. Squares represent the response of the indicated subset for a single individual. Partially visible black squares indicate that the response of CD45RO⁻ cells (■) was essentially identical to the response of CD45RO⁺ cells (□), and that the open square is plotted over the black square.

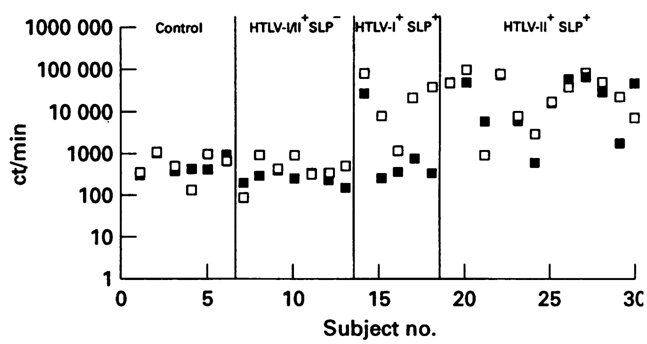

Fig. 3. Spontaneous proliferation (SP) activity of CD45RO⁻ and CD45RO⁺ subsets of CD8 cells. The plotting scheme is identical to that described for Fig. 2.

I/II⁺ SLP⁻ groups (data not shown). With one exception (subject 13, CD45RO⁻ CD4), HTLV-I/II⁺ SLP⁻ individuals did not show SP in either CD4 subset.

A similar analysis of CD45RO⁺ and CD45RO⁻ subsets of CD8 cells demonstrated that four of five HTLV-I⁺ SLP⁺ individuals tested showed SP of the CD45RO⁺ subset, but not the CD45RO⁻ subset; one subject in this group (no. 14) showed SP of both subsets (Fig. 3). In contrast, 10 of 12 individuals in the HTLV-II⁺ SLP⁺ group showed SP in both the CD45RO⁺ and CD45RO⁻ susbsets of CD8 cells; subject 21 showed SP of only the CD45RO⁻ subset, and subject 24 showed SP of only the CD45RO⁺ subset.

Table 2 presents individual subject results from PCR studies assessing the presence of proviral genome in CD45RO⁺ and CD45RO⁻ populations of CD4 cells and CD8 cells from a subset of HTLV-infected individuals. The results demonstrate that, for each of the four cell subsets tested, some infected individuals possessed cells of that subset which were SP⁻ but PCR⁺. Thus, an inability of CD45RO⁺ and CD45RO⁻ cell subsets to undergo SP did not simply reflect the absence of infection for any of the subsets studied. However, SP activity was only observed in association with the presence of provirus.

DISCUSSION

Due to the apparent associaton between T cell activation and efficiency of infection by retroviruses, it has been hypothesized that memory T cells may prove to be easier targets for retroviral

Table 2. Relationship of presence of provirus to spontaneous proliferation (SP) activity—individual subject results

			PCR result (SP activity)			
			CD4 cells		CD8 cells	
Group	Identification no.	SLP status (PBMC)	CD45RO⁻	CD45RO⁺	CD45RO⁻	CD45RO⁺
Control	1	−	ND(−)	ND(−)	−(−)	−(−)
	2	−	−(−)	−(−)	−(−)	−(−)
	3	−	−(−)	−(−)	−(−)	−(−)
HTLV-I	7	−	−(−)	+(−)	+(−)	+(−)
	17	+	+(−)	+(+)	+(−)	+(+)
	18	+	+(−)	+(+)	ND(−)	ND(+)
HTLV-II	8	−	−(−)	+(−)	+(−)	+(−)
	9	−	−(−)	+(−)	+(−)	+(−)
	10	−	−(−)	−(−)	−(−)	ND(−)
	11	−	−(−)	−(−)	+(−)	+(−)
	22	+	+(−)	+(−)	+(+)	+(+)
	23	+	−(−)	−(−)	+(+)	+(+)
	24	+	+(−)	+(−)	+(−)	+(+)
	25	+	+(+)	+(−)	+(+)	+(+)
	26	+	ND(+)	+(+)	+(+)	+(+)
	27	+	+(+)	+(+)	+(+)	ND(+)
	28	+	+(−)	+(−)	+(+)	ND(+)
	29	+	−(−)	−(−)	+(+)	ND(+)
	30	+	+(+)	+(+)	+(+)	ND(+)

ND, Not done; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells.

infection [25]. This line of reasoning led us to hypothesize that SLP in HTLV infection, previously associated with increased viral load [26,27], may occur preferentially in memory cells. Although the data presented here support this hypothesis for HTLV-I infection, they do not support this hypothesis for HTLV-II infection. We found that HTLV-I-associated SLP most often involved only the $CD45RO^+$ memory cell subset of both CD4 cells and CD8 cells. HTLV-II-associated SLP, in contrast, usually involved both the $CD45RO^+$ and $CD45RO^-$ subsets of lymphocytes.

As mentioned earlier, the $CD45RO^+$ cell populations used in these experiments contained both $CD45RO^{dim}$ and $CD45RO^{bright}$ cell subsets, due to the method of cell selection used. We were thus not able to discriminate the SLP capacity of $CD45RO^{dim}$ *versus* $CD45RO^{bright}$ subsets of CD4 cells and CD8 cells. Experiments utilizing cell subsets selected on the basis of CD45RA expression, or perhaps other memory cell markers such as CD29, are thus required to determine if differences in SLP capcity exist for $CD45RO^{bright}$ *versus* $CD45RO^{dim}$ cell subsets.

Results from PCR analysis showed that the lack of SP activity of $CD45RO^+$ and $CD45RO^-$ subsets of CD4 cells and CD8 cells did not merely reflect the absence of proviral genome. These findings confirm and extend our previously published findings of proviral genome presence in CD4 cell and CD8 cell preparations from SLP^- HTLV-infected individuals. They also support the findings of Richardson *et al.* [25], who showed that provirus was present in both $CD45RO^+$ and $CD45RO^-$ lymphocyte subsets from HTLV-I-infected individuals, albeit at higher levels in $CD45RO^+$ cells compared with $CD45RO^-$ cells.

The major finding to be emphasized in this study is that SLP in HTLV-I infection involved only $CD45RO^+$ memory cell subsets of CD4 cells and CD8 cells, whereas SLP in HTLV-II infection involved both $CD45RO^+$ memory and $CD45RO^-$ naive cell subsets of CD4 cells and CD8 cells. The molecular basis for this difference in SP activity of $CD45RO^-$ lymphocytes in HTLV-I *versus* HTLV-II infection remains to be elucidated. Because SP capacity has been linked to proviral load [26,27], clues to this disparity may be provided by studies quantifying the relative proviral load of $CD45RO^+$ and $CD45RO^-$ T cell subsets in HTLV-I and HTLV-II infection. As previously mentioned, Richardson *et al.* [25] have shown that the frequency of infected cells is much higher in $CD45RO^+$ cells than in $CD45RO^-$ cells for HTLV-I infection. To our knowledge, however, such quantification of proviral load in $CD45RO^+$ *versus* $CD45RO^-$ lymphocyte subsets has not been performed for HTLV-II infection. If subsequent studies do indeed demonstrate similar frequencies of infected cells in $CD45RO^+$ and $CD45RO^-$ lymphocyte subsets in HTLV-II infection, they would suggest that no bias toward preferential infection of $CD45RO^+$ cells exists for HTLV-II infection, in contrast to HTLV-I infection. If substantiated, such differences in lymphocyte subset tropism may also shed light on the mechanisms responsible for differences in disease induction in HTLV-I *versus* HTLV-II infection.

An important next step in the characterization of the significance of SP in HTLV disease will be to define the functional capacities of SP^+ memory and naive T cell subsets. Such studies include defining the cytokine production profile and activation marker expression pattern of SP^+ cell subsets, as well as determining if SP^+ T cell subsets possess HTLV-specific cytoxotic activity.

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